USE OF AN ENTEROBACTERIUM OmpA PROTEIN COMBINED WITH AN
ANTIGEN, FOR GENERATING AN ANTIVIRAL, ANTIPARASITIC OR
ANTITUMOR CYTOTOXIC RESPONSE

5   The invention relates to the use of an enterobacterium,
in particular *Klebsiella pneumoniae*, OmpA membrane
protein, combined with an antigen or a hapten, for
preparing a pharmaceutical composition intended to
generate or increase a cytotoxic T response directed
10   against an infectious agent or a tumor cell. The
invention comprises the use of these compounds for
preventing and treating infection or cancer, in
particular cancers combined with a tumor antigen, such
as melanomas, and also for pharmaceutical compositions
15   comprising some of these compounds.

Immunization is an effective means of preventing or
reducing viral or bacterial infections. The success of
immunization campaigns in these domains has made it
20   possible to extend the vaccine concept, until now used
in the domain of infectology, to the domains of cancer
and of autoimmune diseases. Immunization antigens
administered alone to the host are often not
immunogenic enough to induce an immune response and
25   must, therefore, be combined with an adjuvant or
coupled to a carrier protein in order to induce (or
increase) the immunogenicity. Under these conditions,
only an immune response of the humoral type can be
induced. Now, in the context of antiviral therapy, the
30   generation of cytotoxic T lymphocytes (CTLs) capable of
recognizing and destroying the virus is of great
importance (Bachmann et al., 1994, Eur. J. Immunol.,
24, 2228-2236; Borrow P., 1997, J. Virol. Hepat., 4,
16-24), as attested by many studies showing, *in vivo*,
35   the protective role of responses directed against viral
epitopes (Arvin AM, 1992, J. Inf. Dis., 166, S 35-S41;
Koszinowski et al., 1987 Immunol. Lett., 16, 185-192).

The importance of CTL responses has also been greatly documented in antitumor responses, in particular those directed against melanoma cells (review in Rivoltini et al., 1998, Crit. Rev. Immunol. 18, 55-63). The CTL

5 epitope(s) (peptide sequences which interact with class I molecules and are presented to CD8+ T lymphocytes) have been defined for several antigens. However, the difficulty lies in generating CTLs *in vivo*, due to the weak immunogenicity of these peptides (Melief, 1992,

10 Adv. Cancer Res., 58, 143-175; Nandaz and Sercaz, 1995, Cell, 82, 13-17).

Research is consequently directed toward identifying novel adjuvants, or an antigen delivery system, making

15 it possible to induce CTLs. Due to their effectiveness in presenting antigens and in stimulating the immune system, dendritic cells, for example, have been used to generate antiviral CTL responses (Ludewig B et al., 1998, J. Virol., 72, 3812-3818; Brossard P. et al.,

20 1997, J. Immunol., 158, 3270-3276) or anticancer CTL responses (Nestle F.O. et al., 1998, Nat. Med., 4, 328-332). The approaches have consisted in loading the dendritic cells *ex vivo*, with the antigen of interest (peptides or cell lysate) and reimplanting these cells

25 into the patient. Other approaches consist in transfecting, *ex vivo*, the dendritic cells with the gene encoding the antigen of interest and in reinjecting these transfected cells (Gilboa E. et al., 1998, Cancer Immunol. Immunother., 46, 82-87). These

30 approaches have been used successfully in mice and recently in humans (Hsu F.J. et al., 1996, Nat. Med., 2, 52-58), but nevertheless remain complex since the cells must be treated *ex vivo* (transformation of the cells or internalization of the antigens) and

35 transplanted into the host organism. Similarly, the use of viral-type particles (Layton G.T. et al., 1993, J. Immunol., 151, 1097-1107) or of incomplete Freund's adjuvant (IFA) (Valmori et al., Eur. J. Immunol., 1994, 24, 1458-1462) makes it possible to generate CTL

responses. However, antiviral or antitumor immunization carried out with peptides corresponding to CTL epitopes and in the presence of such an adjuvant may lead to a state of specific tolerance, which may, in certain cases, produce the opposite effect to that desired, i.e. a decrease in the immune response (Toes et al., Proc. Nat. Acad. Sci. USA, 1996, 93, 7855-7860).

Thus, there exists, today, a great need for a compound which, when combined with a molecule, in particular an antigen or hapten, is capable of generating CTLs directed against said molecule. Such a compound could, in particular, be used for preparing an immunization composition intended to induce immune protection of the antiviral, antibacterial, antifungal, antiparasitic or antitumor CTL type.

Surprisingly, it has been demonstrated that an outer membrane protein of a gram-negative bacterium, in particular an enterobacterium OmpA protein such as the *Klebsiella pneumoniae* P40 protein (protein described in WO 95/27787 and WO 96/14415), has the property of eliciting a CTL response against a molecule which is covalently or noncovalently associated with it, preferably without having to add another adjuvant.

Thus, the present invention relates to the use of an enterobacterium OmpA protein, of a fragment thereof or of a nucleic acid sequence encoding said OmpA protein or a fragment thereof, for preparing a pharmaceutical composition intended to generate or increase a cytotoxic T response against an infectious agent or a tumor cell, *in vitro* or *in vivo*, preferably *in vivo*, and also for preparing a pharmaceutical composition intended to generate or increase said cytotoxic T response.

In the present invention, the term "protein" is intended to denote both peptides or polypeptides and

the term "OmpA" (for "outer membrane protein") is
intended to denote outer membrane proteins of the A
type.

5   The expression "fragment of an OmpA protein" is
intended to denote, in particular, any fragment of
amino acid sequence included in the amino acid sequence
of the OmpA protein which, when it is combined with an
antigen or hapten specific for an infectious agent or
10   for a tumor cell, is capable of generating or
increasing a cytotoxic T response directed against said
infectious agent or said tumor cell, said fragment of
the OmpA protein comprising at least 5 amino acids,
preferably at least 10 amino acids or more preferably
15   at least 15 amino acids.

The expression "antigen or hapten specific for an
infectious agent or for a tumor cell" is intended to
denote, in particular, any compound expressed by an
20   infectious agent, such as a virus, a bacterium, a
yeast, a fungus or a parasite, or by a tumor cell, or a
structural analog thereof, which, alone or in
combination with an adjuvant of immunity, is capable of
inducing an immune response specific for said
25   infectious agent or for said tumor cell.

In the present description, the expression "analog of
an antigen or hapten" is intended to denote, in
particular, a compound having structural similarity
30   with said antigen or hapten, capable of inducing an
immunological response directed against said antigen or
hapten in an organism immunized beforehand with said
similar compound.

35   A subject of the invention is also the use as claimed
in the invention, characterized in that said
pharmaceutical composition also comprises, combined
with said enterobacterium OmpA protein, an antigen or a

hapten specific for said infectious agent or for said tumor cell.

Preferably, the invention comprises the use as claimed in the invention, characterized in that said infectious agent is a viral particle, a bacterium, a yeast, a fungus or a parasite.

In a particular embodiment, the invention comprises the use of an enterobacterium OmpA protein, or of a fragment thereof, as claimed in the invention, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained using a method of extraction from a culture of said enterobacterium.

The methods for extracting bacterial membrane proteins are known to those skilled in the art and will not be developed in the present description. Mention may, for example, be made, but without being limited thereto, of the extraction method described by Haeuw J.H. et al. (Eur. J. Biochem, 255, 446-454, 1998).

In another preferred embodiment, the invention also comprises the use of an enterobacterium OmpA protein, or of a fragment thereof, as claimed in the invention, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained via the recombinant route.

The methods for preparing the recombinant proteins are, today, well known to those skilled in the art and will not be developed in the present description; reference may however be made to the method described in the examples. Among the cells which may be used for producing these recombinant proteins, mention should, of course, be made of bacterial cells (Olins P.O. and Lee S.C., 1993, Recent advances in heterologous gene expression in E. coli. Curr. Op. Biotechnology

4:520-525), and also yeast cells (Buckholz R.G., 1993, Yeast Systems for the Expression of Heterologous Gene Products. Curr. Op. Biotechnology 4:538-542), as well as animal cells, in particular mammalian cell cultures

5 (Edwards C.P. and Aruffo A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4:558-563), and also insect cells in which methods may be used which implement, for example, baculoviruses (Luckow V.A., 1993, Baculovirus systems

10 for the expression of human gene products. Curr. Op. Biotechnology 4:564-572).

Entirely preferably, the use as claimed in the invention is characterized in that said enterobacterium

15 is *Klebsiella pneumoniae*.

In particular, the invention relates to the use as claimed in the invention, characterized in that the amino acid sequence of said *Klebsiella pneumoniae* OmpA

20 protein, or a fragment thereof, comprises:

a)   the amino acid sequence of sequence SEQ ID No. 2;
b)   the amino acid sequence of a sequence having at least 80%, preferably 90% and 95% homology, after

25   optimal alignment, with the sequence SEQ ID No. 2; or

c)   the amino acid sequence of a fragment of at least 5 amino acids, preferably 10, 15, 20 and 25 amino acids, of a sequence as defined in a).

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The expression "nucleic acid or amino acid sequence having at least 80% homology, after optimal alignment, with a given nucleic acid or amino acid sequence" is intended to denote a sequence which, after optimal

35 alignment with said given sequence, comprises a percentage identity of at least 80% with said given sequence.

For the purposes of the present invention, the term
"percentage identity" between two nucleic acid or amino
acid sequences is intended to denote the percentage of
nucleotides or of amino acid residues which are
5   identical between the two sequences to be compared,
obtained after the best alignment, this percentage
being purely statistical and the differences between
the two sequences being distributed randomly and over
their entire length. Sequence comparisons between two
10   nucleic acid or amino acid sequences are conventionally
carried out by comparing these sequences after having
aligned them optimally, said comparison being carried
out by segment or by "window of comparison" in order to
identify and compare local regions of sequence
15   similarity. The optimal alignment of the sequences for
comparison may be produced, other than manually, by
means of the local homology algorithm of Smith and
Waterman (1981) [Ad. App. Math. 2:482], or by means of
the local homology algorithm of Neddleman and Wunsch
20   (1970) [J. Mol. Biol. 48:443], or by means of the
similarity search method of Pearson and Lipman (1988)
[Proc. Natl. Acad. Sci. USA 85:2444], or by means of
computer software which uses these algorithms (GAP,
BESTFIT, FASTA and TFASTA in the Wisconsin Genetics
25   Software Package, Genetics Computer Group, 575 Science
Dr., Madison, WI, or with BLAST N or BLAST P comparison
software).
The percentage identity between two nucleic acid or
amino acid sequences is determined by comparing these
30   two sequences which are optimally aligned by the window
of comparison in which the region of the nucleic acid
or amino acid sequence to be compared may comprise
additions or deletions with respect to the reference
sequence for optimal alignment between these two
35   sequences. The percentage identity is calculated by
determining the number of identical positions for which
the nucleotide or the amino acid residue is identical
between the two sequences, dividing this number of
identical positions by the total number of positions in

the window of comparison and multiplying the result obtained by 100 in order to obtain the percentage identity between these two sequences.

5 Use may, for example, be made of the BLAST program "BLAST 2 sequences", which is available on the site http://www.ncbi.nlm.nih.gov/gorf/bl2.html, the parameters used being those given by default (in particular for the "open gap penalty" parameter: 5, and
10 the "extension gap penalty" parameter: 2; the matrix chosen being, for example, the "BLOSUM 62" matrix provided by the program), the percentage identity between the two sequences to be compared being calculated directly by the program.

15

Among said sequences having at least 80% homology with the reference OmpA sequence, preference is given to the sequences of, or encoding, peptides capable of inducing CTL activity directed specifically against the antigen
20 or hapten which is combined with it, such as the CTL activity measured using the standard techniques described in the examples hereinafter.

The invention also comprises the use as claimed in the
25 invention, characterized in that said antigen or hapten is chosen from proteins, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids or any compound capable of specifically directing the CTL response against said infectious agent or said tumor cell.
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A subject of the present invention is also the use as claimed in the invention, characterized in that said antigen or hapten is coupled to or mixed with said OmpA protein or a fragment thereof.

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The invention also comprises the use as claimed in the invention, characterized in that said antigen or hapten is coupled by covalent attachment, in particular by

chemical coupling, with said OmpA protein or a fragment thereof.

In a particular embodiment, the use as claimed in the invention is characterized in that one or more attachment elements is(are) introduced into said OmpA protein, or a fragment thereof, and/or into said antigen or hapten, in order to facilitate the chemical coupling; preferably, said attachment element introduced is an amino acid.

As claimed in the invention, it is possible to introduce one or more attachment elements, in particular amino acids, in order to facilitate the coupling reactions between the OmpA protein, or a fragment thereof, and said antigen or hapten. The covalent coupling between the OmpA protein, or a fragment thereof, and said antigen or hapten as claimed in the invention may be carried out at the N- or C-terminal end of the OmpA protein or a fragment thereof. The difunctional reagents which enable this coupling will be determined as a function of the end of the OmpA protein, or a fragment thereof, which is chosen for carrying out the coupling, and of the nature of said antigen or hapten to be coupled.

In another particular embodiment, the use as claimed in the invention is characterized in that the coupling between said antigen or hapten and said OmpA protein, or a fragment thereof, is produced by genetic recombination, when said antigen or hapten is peptide in nature.

The conjugates derived from coupling to said OmpA protein, or a fragment thereof, may be prepared by genetic recombination. The chimeric or hybrid protein (conjugate) may be produced using recombinant DNA techniques, by inserting or adding a sequence encoding said antigen or hapten which is peptide in nature into

the DNA sequence encoding said OmpA protein or a fragment thereof.

The methods for synthesizing the hybrid molecules encompass the methods used in genetic engineering for constructing hybrid polynucleotides encoding desired polypeptide sequences. Advantageously, reference may, for example, be made to the technique for obtaining genes encoding fusion proteins, described by D.V. Goeddel (Gene expression technology, Methods in Enzymology, Vol. 185, 3-187, 1990).

In another aspect, the invention relates to the use as claimed in the invention, characterized in that the pharmaceutical composition comprises a nucleic acid construct encoding said hybrid protein, or comprises a vector containing a nucleic acid construct encoding said hybrid protein or a transformed host cell containing said nucleic acid construct, which is capable of expressing said hybrid protein.

The invention also comprises the use as claimed in the invention, for preparing a pharmaceutical composition intended to eliminate infectious agents or inhibit tumor growth.

Preferably, the use as claimed in the invention relates to the preparation of a pharmaceutical composition intended to prevent or treat infectious diseases or cancers, preferably cancers associated with a tumor antigen.

Among cancers in which the tumors express an associated tumor antigen, and which may be prevented or treated with the uses as claimed in the present invention, mention may be made, in particular, but without being limited thereto, of:

- breast cancer, lung cancer, colon cancer and gastric carcinoma (Kawashima et al., 1999, Cancer Res. 59:431-5);

- mesothelioma, osteosarcoma, brain cancers (Xie et al., 1999, J. Natl. Cancer. Inst. 91:169-75);

- melanoma (Zheuten et al., 1998, Bratilsl. Lek. Listy 99:426-34);

- cystic adinoma of the pancreas (Hammel et al., 1998, Eur. J. gastroenterol. Hepatol. 10:345-8);

- colorectal cancer (Ogura et al., 1998, Anticancer Res. 18:3669-75);

- renal cell carcinoma (Jantzer et al., 1998, Cancer Res. 58:3078-86); and

- cancer of the ovary and of the cervix (Sonoda et al., 1996, Cancer. 77:1501-9).

A subject of the invention is in particular the use of an enterobacterium OmpA protein, or of a fragment thereof, as claimed in the invention, for preparing a pharmaceutical immunization composition intended to prevent or treat an infectious disease, in particular of viral, bacterial, fungal or parasitic origin, or a cancer, preferably associated with a tumor antigen, in particular melanomas.

The invention also comprises the use as claimed in the invention, characterized in that said pharmaceutical composition is vehicled in a form which makes it possible to improve its stability and/or its immunogenicity, in particular in the form of a liposome.

Preferably, the invention comprises the use as claimed in the invention, characterized in that said vehicle is a viral vector containing a nucleic acid construct encoding said OmpA protein or a fragment thereof, said antigen or hapten, or said hybrid protein, or a transformed host cell capable of expressing said OmpA

protein or a fragment thereof, said antigen or hapten, or said hybrid protein.

The invention also comprises the use as claimed in the invention, characterized in that said nucleic acid construct, or the nucleic acid construct contained in said vector or said transformed host cell, comprises a nucleic acid sequence chosen from the sequence SEQ ID No. 1, a fragment thereof having at least 15 consecutive nucleotides, preferably 20, 25, 30, 40 and 50 consecutive nucleotides, of the sequence SEQ ID No. 1, or a sequence having at least 80%, preferably 90% and 95%, homology, after optimal alignment, with one of said sequences.

In another aspect, the invention comprises a pharmaceutical composition as defined above in the uses as claimed in the present invention.

Among these compositions, preference is given to the pharmaceutical compositions characterized in that they comprise, in a pharmaceutically acceptable medium, at least one enterobacterium OmpA protein, or a fragment thereof, combined, by mixing or by coupling, with at least one antigen or one hapten associated with or specific for a tumor cell.

For the purposes of the present invention, the pharmaceutically acceptable medium is the medium in which the compounds of the invention are administered, preferably a medium which can be injected into humans. It may consist of water, of an aqueous saline solution or of an aqueous solution based on dextrose and/or on glycerol.

In one particular embodiment, the composition as claimed in the invention also contains a detergent.

The compositions as claimed in the invention may also contain a detergent, and in particular any type of pharmaceutically acceptable surfactant, such as for example anionic, cationic, nonionic or amphoteric surfactants. Use is preferably made of the detergents Zwittergent 3-12 and octylglucopyrannoside, and even more preferably Zwittergent 3-14.

Preferably, the pharmaceutical composition as claimed in the invention is characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained using a method of extraction from a culture of said enterobacterium or via the recombinant route.

Again preferably, the pharmaceutical composition as claimed in the invention is characterized in that said enterobacterium is Klebsiella pneumoniae.

In a preferred embodiment, the invention relates to a composition as claimed in the invention, characterized in that the aminoacid sequence of said OmpA protein, or a fragment thereof, comprises:

a) the amino acid sequence of sequence SEQ ID No. 2;

b) the amino acid sequence of a sequence having at least 80% homology with the sequence SEQ ID No. 2; or

c) the amino acid sequence of a fragment of at least 5 amino acids, preferably 10, 15, 20 and 25 amino acids, of a sequence as defined in a).

Among the antigens or haptens which are part of the composition as claimed in the invention, preference is give to those chosen from peptides, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids or any compound capable of specifically directing a CTL response against a tumor cell.

In an equally preferred embodiment, the invention relates to a composition as claimed in the invention,

characterized in that said antigen or hapten is coupled, by covalent attachment, with said OmpA protein, or a fragment thereof, in particular by coupling produced by chemical synthesis and for which,

5 where appropriate, one or more attachment elements, such as an amino acid, may be introduced into said OmpA protein, or a fragment thereof, and/or into said antigen or hapten, in order to facilitate said chemical coupling.

10

In an equally preferred embodiment, the invention relates to a composition as claimed in the invention, characterized in that the coupling between said antigen or hapten and said OmpA protein, or a fragment thereof,

15 is produced by genetic recombination, when said antigen or hapten is peptide in nature (expression of a hybrid protein).

Thus, the present invention also relates to a

20 composition as claimed in the invention, characterized in that the pharmaceutical composition comprises a nucleic acid construct encoding said hybrid protein, said nucleic acid construct possibly being contained in a vector, or in a transformed host cell capable of

25 expressing said hybrid protein.

In a preferred embodiment, the invention relates to a composition as claimed in the invention, characterized in that said nucleic acid construct comprises a nucleic

30 acid sequence chosen from the sequence SEQ ID No. 1, a fragment thereof having at least 15 consecutive nucleotides of the sequence SEQ ID No. 1, or a sequence having at least 80% homology with one of said sequences.

35

Among the compositions as claimed in the invention, preference is also given to the pharmaceutical compositions vehicled in a form which makes it possible to improve their stability and/or their immunogenicity,

in particular in the form of a liposome, of a viral vector containing a nucleic acid construct encoding said OmpA protein, or a fragment thereof, said antigen or hapten, or said hybrid protein, of of a transformed host cell capable of expressing said OmpA protein, or a fragment thereof, said antigen or hapten, or said hybrid protein.

In a final aspect, the composition as claimed in the invention is characterized in that it contains no other adjuvant for inducing a CTL response, besides said enterobacterium OmpA protein, or a fragment thereof, or a nucleic acid construct encoding said OmpA protein, or a fragment thereof, a characteristic element of the composition as claimed in the invention for inducing a CTL response.

The legends to the figures and examples which follow are intended to illustrate the invention without in any way limiting the scope thereof.

Legends to the figures:

Figures 1A, 1B, 1C and 1D: Measurement of the anti-MELAN-A and anti-TRP-2 CTL activity of effector cells

After immunization with 50 µg of hELA mixed with 3 µg of rP40 (figure 1A), 50 µg of hELA mixed with 300 µg of rP40 (figure 1B), 50 µg of hELA coupled to rP40 (figure 1C) or 50 µg of the TRP-2 peptide mixed with 300 µg of rP40 (figure 1D), the draining lymph mode cells are stimulated with EL-4 A2/Kb cells (figures 1A, 1B and 1C) or EL-4 cells (figure 1D) which had been irradiated and prepulsed with 1 µM of the relevant peptide, before being evaluated for their capacity to kill target cells which may (rectangle) or may not (diamond) have been prepulsed with the relevant peptide.

The X-axes of the points of figures 1A to 1D correspond to the ratio of the effector T cells (active lymphocytes) mixed together with the target cells (EL-4 A2/Kb or EL-4).

Figures 2A, 2B, 2C and 2D: Measurement of the anti-MELAN-A CTL activity of effector cells in the presence of the rP40 protein compared to the CTL activity obtained with standard immunization protocol.

After immunization with hELA (50 µg) alone (ELA, figure 2A), hELA mixed with 300 µg of rP40 (ELA + P40, figure 2B), hELA coupled to 300 µg of rP40 (ELA/P40, figure 2C) or hELA mixed with 50 µg of P30 peptide adjuvanted with IFA (ELA + IFA + TT, figure 2D) (IFA for Incomplete Freund's Adjuvant and TT for Tetanus Toxoid), the draining lymph node cells are stimulated in vitro for two weeks with EL-4 A2/Kb cells which have been irradiated and prepulsed with 1 µM of the relevant peptide, before being evaluated for their capacity to kill EL-4 A2/Kb target cells which may (rectangle) or may not (triangle) have been prepulsed with the hELA peptide.

Figures 3A, 3B, 3C and 3D: CTL activity and antitumor effect of the immunization with rP40 + TRP-2 peptide.

Figure 3A: The immunization with a mixture of the TRP-2 peptide with rP40 induces a CTL response specific for the peptide. C57BL/6 mice were injected subcutaneously with 50 µg of the TRP-2 peptide mixed with 300 µg of rP40. Ten days later, the lymph nodes were dissociated and restimulated with irradiated EL-4 cells which may (rectangles) or may not (diamonds) have been pulsed with the TRP-2 peptide.

Figures 3B, 3C and 3D: C57BL/6 mice received 2×10^3 cells of the B16F10 autologous melanoma, subcutaneously into the flank. Simultaneously (figures 3B and 3D), or 4 days later (figure 3C), some of these mice were

immunized subcutaneously (at the base of the tail) with
50 μg of the TRP-2 peptide mixed with 300 μg of rP40
(o), and others were immunized with the P40 protein
alone (■, for figures 3B and 3C) or with the TRP-2
5 peptide alone (■, for figure 3D). From the 18th day
postimplantation, the volume of the tumors was
measured.

Figures 4A, 4B and 4C: Measurement of the anti-OVA CTL
activity after immunization with the rP40 protein
10 coupled to the p257-264 OVA peptide.

C57BL/6 mice received, by subcutaneous injection at the
base of the tail, 200 μg of P40-Ova (■), of Ova-coupled
beads (O), of solubilized Ova (□), of Ova-BS^3 (▲) (BS^3
15 for bis(succinimidyl) suberate), of P40 (←), or of
DT-Ova (●)(DT for diphtheria toxoid).

The EL4 thymoma target cells pulsed with 50 μg/ml of
OVA peptide (figure 4B) or not pulsed (figure 4C), or
20 transfected with the *ova* gene (E.G7 line) (figure 4A)
are incubated with ^{51}Cr at 37°C and cultured with the
effector cells.

Example 1: Cloning of the gene encoding the *Klebsiella*
25 *pneumoniae* P40 protein

The gene encoding the P40 protein was obtained by PCR
amplification using the genomic DNA of *Klebsiella*
pneumoniae IP I145 (Nguyen et col., Gene, 1998). The
30 gene fragment encoding this gene is inserted into
various expression vectors under the control of various
promoters, in particular that of the Trp operon. The
nucleotide sequence and the peptide sequence of the P40
protein are represented by the sequences SEQ ID No. 1
35 and SEQ ID No. 2 hereinafter. An *E. coli* K12 producer
strain was transformed with a pvaLP40 expression
vector. The recombinant P40 protein (named rP40) is
produced, in the form of inclusion bodies, with a

considerable yield (> 10% in g of protein/g of dry biomass).

This example is merely an illustration of the expression of the rP40 protein, this illustration possible being extended to other bacterial strains and to other expression vectors.

Example 2: Method for fermentation of rP40 fusion proteins

An Erlenmeyer flask containing 250 ml of TSB (Tryptic Soy Broth, Difco) medium containing ampicilline (100 µg/ml, Sigma) and tetracyclin (8 µg/ml, Sigma) is inoculated with the transformed E. coli strain described above. After overnight incubation at 37°C, 200 ml of this culture are used to seed 2 liters of culture medium in a fermenter (Biolaffite, France). In a quite conventional way, the culture medium may be composed of chemical agents supplemented with vitamins and/or yeast extracts, which are known to promote high density bacterial cell growth.

The parameters controlled during the fermentation are: pH, stirring, temperature, level of oxygenation and supply of combined sources (glycerol or glucose). In general, the pH is regulated at 7.0 and the temperature is fixed at 37°C. The growth is controlled by supplying glycerol (87%) at a constant rate (12 ml/h) in order to maintain the dissolved oxygen tension signal at 30%. When the turbidity of the culture (measured at 580 nm) reaches the value of 80 (after culturing for approximately 24 hours), the protein production is treated by adding indole acrylic acid (IAA) at the final concentration of 25 mg/l. Approximately 4 hours after induction, the cells are harvested by centrifugation. The amount of wet biomass obtained is approximately 200 g.

Example 3: Method for extracting and purifying the rP40 protein

Extracting the rP40

5 After centrifugation of the culture broth (4000 rpm (revolutions per minute), 10 min, 4°C), the cells are resuspended in a 25 mM Tris-HCl buffer, pH 8.5. The insoluble components, or inclusion bodies, are obtained after treatment with lysozyme (0.5 g/liter, 1 hour at
10 room temperature with gentle stirring). The inclusion body pellet obtained by centrifugation (15 min at 10,000 g at 4°C) is taken up in a 25 mM Tris-HCl buffer at pH 8.5 containing 5 mM $MgCl_2$ and then centrifuged (15 min at 10,000 g).

15

The inclusion bodies are solubilized at 37°C for 2 hours in a 25 mM Tris-HCl buffer, pH 8.5, containing 7 M urea (denaturing agent) and 10 mM of dithiothreitol (reduction of disulfide bridges). Centrifugation
20 (15 min at 10,000 g) makes it possible to eliminate the insoluble particles.

This is then followed by resuspension in 13 volumes of 25 mM Tris-HCl buffer, pH 8.5, containing NaCl
25 (8.76 g/l) and Zwittergent 3-14 (0.1%, w/v). The solution is left overnight at room temperature with gentle stirring in contact with the air (to promote renaturation of the protein by dilution and reoxidation of the disulfide bridges).

30

Purifying the rP40 protein

-    Anion exchange chromatography step

35 After a further centrifugation, the solution is dialyzed against a 25 mM Tris-HCl buffer, pH 8.5, containing 0.1% Zwittergent 3-14 (100 volumes of buffer) overnight at 4°C.

The dialyzate is loaded on to a column containing a support of the strong anion exchanger type (Biorad Macro Prop High Q gel) equilibrated in the buffer described above, at a linear flow rate of 15 cm/h. The

5   proteins are detected at 280 nm. The rP40 protein is eluted, with a linear flow rate of 60 cm/h, for an NaCl concentration of 0.2 M in the 25 mM Tris-HCl buffer, pH 8.5: 0.1% Zwittergent 3-14.

10   -   Cation exchange chromatography step

The fractions containing the rP40 protein are pooled and concentrated by ultrafiltration with the aid of an Amicon cell system with stirring, used with a YM10-type

15   Diaflo membrane (10 kDa cut-off threshold), for volumes of about 100 ml, or with the aid of a Millipore Minitan tangential flow filtration system, used with membrane plates having a 10 kDa cut-off threshold, for larger volumes. The fraction thus concentrated is dialyzed

20   overnight at 4°C against a 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 3-14.

The dialyzate is loaded on to a column containing a support of the strong cation exchanger type (Biorad

25   Macro Prep High S gel) equilibrated in the 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 3-14. The rP40 protein is eluted (rate 61 cm/h) for a 0.7 M NaCl concentration. The electrophoretic profiles show about a 95% degree of purity. The condition of the

30   protein is monitored by SDS-PAGE. The P40 protein, extracted from the *Klebsiella pneumoniae* membrane, has a characteristic electrophoretic (migration) behavior depending on whether it is in denatured or native form. The native form (β-sheet structure) in fact has a lower

35   molecular mass than the form which is denatured (α-helical structure) by the action of a denaturing agent, such as urea or guanidine hydrochloride, or by heating at 100°C in the presence of SDS. The rP40 protein is not properly renatured at the end of renaturation,

regardless of whether the latter is carried out in the presence or absence of 0.1% (w/v) Zwittergent 3-14. On the other hand, total renaturation is obtained after dialysis against a 25 mM Tris/HCl buffer, pH 8.5,

5   containing 0.1% (w/v) Zwittergent 3-14. However, it should be noted that this renaturation is only obtained when the dilution step and treatment at room temperature are, themselves, carried out in the presence of Zwittergent 3-14 (negative results in the

10   absence of detergent).

Example 4: Generation of CTLs

The antitumor CTL responses directed against melanoma

15   cells were defined for several antigens. These antigens are included in one of three categories:

a)   rejection antigen specific for melanoma, such as those of the MAGE family (review by van der Bruggen et

20   al., Science 254:1643);

b)   antigens resulting from the mutation of normal proteins. This group includes MUM-1 (Coulie et al., Proc. Natl. Acad. Sci. USA 92:7976-7980 (1995)); CDK4 (Wolfel et al., Science 296:1281-1284 (1995)) and HLA-

25   A2 (Brandel et al., J. Exp. Med. 183:2501-2508 (1996));

c)   differentiation antigens expressed by melanomas and melanocytes. This group includes tyrosinase (Wolfel et al., Eur. J. Immunol. 4:759 (1994) and Brichard et al., Eur. J. Immunol. 26:224 (1996)); gp 100 (Kang et

30   al., J. Immunol. 155:1343 (1995), Cox et al., Science 264:716 (1994), and Kawakami et al., J. Immunol. 155:3961 (1995)); gp75 (Wang et al., J. Exp. Med. 183:1131 (1996)), and Mart-1/MelanA (see US patent 5,620,886).

35

Of all these antigens, Mart-1/MelanA appears to be the best candidate for use in immunotherapy, this being for several reasons. Firstly, this antigen was identified on the basis of the CTL response, in vivo, of the

lymphocytes infiltrating the melanoma and not that, in vitro, of the peripheral blood cells, which would suggest greater relevance of this antigen in the natural response, in vivo, against melanoma (Kawakami

5 et al., J. Exp. Med. 180:347 (1994)). In addition, Mart-1/MelanA is expressed on all melanomas examined, which makes it a preferred target for intervention by immunotherapy. Finally, peptides derived from Mart-1/MelanA are capable of inducing a specific CTL

10 response in patients with melanoma expressing the HLA-A2 histocompatibility antigen (Rivoltini et al., J. Immunol. 154:2257 (1995); Valmori et al., J. Immunol. 160:1750 (1998)).

15 HLA-A2 is the most common allele expressed in Caucasians. The CTL epitopes of Mart-1/MelanA have been defined for this allele. The antigenic peptide recognized by the majority of human CTL lines comprises amino acids 27-35 AAGIGILTV (Kawakami et al., J. Exp.

20 Med. 180:347 (1994)). In addition, studies on the affinity of binding with HLA-A*0201 and recognition by CTL clones have demonstrated that the optimum peptide for these two functions is the 26-35 decapeptide EAAGIGILTV (Romero et al., J. Immunol. 159:2366

25 (1997)). However, it appears that these peptides are weakly immunogenic in vitro (Valmori et al., J. Immunol. 160:1750 (1998)) and in vivo (Jaeger et al., Int. J. Cancer 66:162 (1996)).

30 When comparing the amino acid sequence of the T epitopes of Mart-1/MelanA with the peptide motifs of A*0201 (Rammensee et al., Immunogenetics 41:178 (1995)), it appears that the 26-35 and 27-35 peptides have nondominant anchoring residues at position 2 and

35 therefore weakly bind the HLA-A*0201 molecule (Kawakami et al., J. Immunol. 154:3961 (1995)), which might explain their weak immunogenicity. The international patent application published under the number WO 98/58951 describes an analog to the 26-35 peptide,

in which the alanine at position 2 has been replaced
with a leucine (sequence which will be named ELA of
sequence SEQ ID No. 3).

5   The hELA peptide, used in the experiments below, is the
subject of patent application WO 98/58951 which is the
property of the Institut Ludwig de Recherche sur le
Cancer [Ludwig Cancer Research Institute]. hELA is an
analog of the 26-35 decapeptide (EAAGIGILTV) of
10   Melan-A/MART-1, which is a protein expressed on
melanocytes and melanomas. Although the 26-35
decapeptide of Melan-A/MART-1 is capable of binding to
the HLA-A0201 molecule (Romero et al., 1997, J.
Immunol. 159, 2366-2374), it is weakly immunogenic
15   in vitro and in vivo (Valmori et al., 1998, J. Immunol.
160, 1750-1758). The hELA analog was generated by
substituting the second amino acid of the 26-35
decapeptide of Melan-A/MART-1 (an alanine) with a
leucine. The result of this substitution, which is
20   based on analysis of the residues required for
anchoring the peptides to the HLA-A0201 molecule, is
more effective recognition by the CTLs of patients with
melanoma and better immunogenicity in vitro (Valmori
et al., 1998, J. Immunol. 160, 1750-1758).
25   HLA-A*0201/Kb (A2/Kb) transgenic mice of the strain
C57Bl/6 x BDA/2 (Vitiello et al., 1991, J. Exp. Med.,
173, 1007-1015) were used in this study to test ELA.
The class I MHC molecule expressed in these mice is a
chimeric molecule made from the α1 and α2 domains of
30   the human HLA-A0201 molecule (the most common allotype
found) and from the α3 domain of the murine K^b
molecule.

The TRP-2 peptide of sequence SEQ ID No. 4 is an
35   octapeptide corresponding to amino acids 181-188
(VYDFFVWL) of tyrosinase-related protein 2 (TRP-2).
TRP-2 is expressed in melanocytes and melanomas. It has
been demonstrated that this antigen induces CTL
responses which protect against melanoma in C57BL/6

($H-2K^b$) mice (Bloom et al., 1997, J. Exp. Med. 185, 453-459).

A: Generation of anti-Melan-A and anti-TRP-2 CTLs after immunization with rP40 mixed with a peptide which is an analog to Melan-A or TRP-2

Experimental protocol

A2/Kb mice received, by subcutaneous injection at the base of the tail:
- 50 μg of ELA mixed with 3 or 300 μg of rP40;
- 50 μg of ELA covalently coupled to 300 μg of rP40.

C57BL/6 mice received, by subcutaneous injection into the base of the tail:
- 50 μg of the TRP-2 peptide (181-188) mixed with 300 μg of rP40.

Generation of cytotoxic effector cells

10 days after immunization, the mice are sacrificed and the lymphocytes from the draining lymph nodes are recovered in order to be stimulated, in vitro, with the relevant peptide.

These lymphocytes (4 to 5 × 10^6) are cultured in a 24 well plate in DMEM plus 10 mM HEPES, 10% FCS and 50 μM β-2-mercaptoethanol, with 2 to 5 × 10^5 EL-4 A2/Kb cells or EL4 cells which have been irradiated (10 kRads) and prepulsed for 1 h at 37°C with 1 μM of the relevant peptide. After two weekly stimulations, the cells are assayed for their cytotoxic activity.

Measurement of cytotoxic activity

The EL-4 A2/Kb cells or EL4 cells are incubated for 1 h with ^{51}Cr in the presence or absence of the relevant peptide, washed and then coincubated with the effector cells at various ratios, in a 96-well plate in a volume

of 200 μl for 4 to 6 h at 37°C. The cells are then centrifuged and the ^{51}Cr release is measured in 100 μl of supernatant. The percentage of specific lysis is calculated as follows:

5   % specific lysis = (experimental release – spontaneous release) / (total release – spontaneous release) X 100.

Results

As shown in figures 1A to 1D, the immunization of mice

10   with an optimal dose of rP40 (300 μg) in a mixture with hELA (figure 1B) or TRP-2 (figure 1D) induces a strong specific CTL response. Such a response is also observed after immunization with rP40 coupled to hELA (figure 1C). On the other hand, the immunization with

15   the peptide alone or rP40 alone (results not shown) or with the hELA peptide in a mixture with a suboptimal dose of rP40 (3 μg) does not induce any CTL activity (figure 1A). These results demonstrate that the rP40 molecule mixed with or coupled to immunogenic peptides

20   makes it possible to induce a specific CTL response in vivo, this being without the addition of adjuvant.

B: Generation of anti-Melan-A CTLs after immunization with rP40 mixed with a peptide which is an analog to

25   Melan-A, compared to a standard immunization protocol
Experimental protocol

A2/Kb mice received:

–   50 μl of IFA (incomplete Freud's adjuvant) by

30   subcutaneous injection at the base of the tail, then, 3 weeks later, 50 μg of hELA in the presence of 50 μg of a helper-T p30 peptide derived from Tetanus Toxoid (TT) (Panina-Bordignon et al., Eur. J. Immunol., 1989, 19, 2237) adjuvanted with IFA. This protocol has been

35   described for generating anti-peptide CTLs (Valmori et al., Eur. J. Immunol., 1994, 24, 1458) and is used as a positive control.

–   50 μg of hELA alone or 300 μg of rP40 mixed with or coupled to 50 μg of hELA.

Generation of cytotoxic effector cells

10 days after the final immunization, the mice are
sacrificed and the lymphocytes from the draining lymph
nodes are recovered in order to be stimulated,
in vitro, with the relevant peptide.

These lymphocytes (4 to 5×10^6) are cultured in a
24-well plate in DMEM plus 10 mM HEPES, 10% FCS and
50 µM β-2-mercaptoethanol, with 2 to 5×10^5 EL-4 A2/Kb
cells (murine cells transfected with the HLA-A* 0201/Kb
gene) which have been irradiated (10 kRads) and
prepulsed for 1 h at 37°C with 1 µM of the relevant
peptide.

After one, two or three weekly stimulations, the cells
are assayed for their cytotoxic activity.

The cytotoxic activity is measured according to the
method described above.

Results

After immunization with nonadjuvanted rP40 coupled to
hELA, an anti-hELA CTL activity comparable to that
observed after immunization with hELA + P30/IFA is
measured (cf. figures 2C and 2D). Similarly, the rP40 +
hELA peptide mixture, itself also nonadjuvanted,
generates CTLs in a way which is similar to that
obtained with a conventional protocol for generating
CTLs (cf. figures 2B and 2D).

No CTL activity was detected after immunization with
the peptide alone (cf. figure 2A) or the rP40 protein
alone (not shown), regardless of the day on which the
effector cells were stimulated.

Example 5: Antitumor effect of the immunization with a mixture of rP40 and of a peptide expressed by a mouse melanoma

5   In order to evaluate the capacity of rP40 to generate an antitumor CTL response, the capacity of the rP40 protein to induce a CTL response directed against the peptide of sequence SEQ ID No. 4 (VYDFFVWL) was tested. The peptide of sequence SEQ ID No. 4 (VYDFFVWL) is

10   derived from Tyrosinase Related Protein 2 (TRP-2) which is expressed by the B16F10 melanoma derived from C57BL/6 mice. This peptide is immunogenic in this strain. The growth of the B16F10 cells implanted into C57BL/6 mice which were or were not immunized with a

15   mixture of rP40 and of the TRP-2 peptide was then tested.

Experimental protocol

20   For generating an anti-TRP-2 peptide CTL response, a protocol identical to that described in example 4 was used, except that, on this occasion, C57BL/6 mice were used.

25   For the protection experiments, C57BL/6 mice received 2×10^3 cells of the B16F10 autologous melanoma, by subcutaneous (s.c.) injection into the flank. Simultaneously, or 4 days later, some of these mice were immunized subcutaneously (at the base of the tail)

30   with 50 μg of the TRP-2 peptide mixed with 300 μg of rP40. The growth of the tumor was then measured at regular intervals.

Results

35

As shown in figure 3A, the immunization with a mixture of TRP-2 peptide and of the RP40 protein is capable of generating a specific CTL response to this peptide, which confirms the results obtained with the hELA

protein (described in example 4). In addition, this CTL response is associated with inhibition of the growth of the B16F10 melanoma (figures 3B, 3C and 3D). It is of value to note that this protection is significant not only when the immunization with the TRP-2 peptide + rP40 is carried out simultaneously with the implantation of the tumor (figures 3b and 3D), but also when carried out 4 days after the implantation (figure 3C).

These results clearly show the therapeutic effect of the use of an enterobacterium OmpA protein, such as the *K. pneumoniae* OmpA protein, combined with an antigenic tumor peptide, in order to induce a specific CTL-type response which is effective in preventing or treating cancer, such as melanomas.

Example 6: Generation of anti-OVA CTLs after immunization with rP40 coupled to the p257-264 Ova peptide

The p257-264 Ova peptide is an octapeptide corresponding to the fragment of the ovalbumin consensus sequence which is between the amino acids at position 257 to 264 of the ovalbumin sequence (ends included). Ovalbumin is used as a peptide which protects against tumor cells expressing ovalbumin.

Experimental protocol

C57BL/6 mice received, by subcutaneous injection into the base of the tail, 200 µg of P40-Ova (□), of Ova-coupled beads (O), of solubilized Ova (■), of Ova-BS^3 (□) (BS^3 for bis(succinimidyl) suberate), of P40 (←) or of DT-Ova (●) (DT for Diphtheria Toxoid).

Generation of cytotoxic effector cells

7 days after immunization, the mice are sacrificed and
the spleens are recovered. The spleen cells (4×10^7)
5   are cultured in flasks, in DMEM with 1.5×10^6
irradiated (4kRads) E.G7 cells.

Measurement of the cytotoxic activity

10   The EL4 thymoma cells which were pulsed or not pulsed
with the OVA peptide or transfected with the *ova* gene
(E.G7 line) are incubated with ^{51}Cr at 37°C and cultured
with the effector cells obtained above.

15   The percentage of specific lysis is calculated as
described in example 4A.

Results

20   As shown in figures 4A to 4C, the immunization of mice
with the rP40 protein coupled to or mixed with the OVA
peptide induces a strong specific CTL response. This
response is similar to that observed after immunization
with the positive control, namely the ovalbumin-coupled
25   beads (see figures 4A and 4B). On the other hand, the
immunization with soluble ovalbumin, ova-BS^3 and DT-Ova
is not effective. These results demonstrate that the
rP40 module coupled to an immunogenic peptide makes it
possible to induce a specific CTL response in vivo,
30   this being without the addition of adjuvant.